Production and Characterization of Monoclonal Antibodies Against the Protective Antigen Component of *Bacillus anthracis* Toxin

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee of Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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Thirty-six monoclonal antibodies to the protective antigen protein of Bacillus anthracis exotoxin have been characterized for affinity, antibody sub-type, competitive binding to antigenic regions, and ability to neutralize the lethal and edema toxin activities. At least 23 antigenic regions were detected on protective antigen by a blocking, enzyme-linked immunosorbent assay. Two clones, 3B6 and 14B7, competed for a single antigenic region and neutralized the activity of both the lethal toxin in vivo (Fisher 344 rat) and the edema toxin in vitro (CHO cells). These two antibodies blocked the binding of ^{125}I-PA to FRL-103 cells. Our results support the proposal that binding of protective antigen to cell receptors is required for expression of toxicity.

Two plasmid-encoded, virulence factors, a capsule and exotoxin (10, 17, 28), have been described for Bacillus anthracis. The exotoxin is composed of three proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF). Protective antigen induces immunity to infection with B. anthracis and is the major component in the commercially prepared, cell-free U.S. and British vaccines (11, 16, 27). Toxic activity is expressed only when PA is combined with EF or LF. A combination of PA with EF (PA+EF) or LF (PA+LF) forms edema toxin or lethal toxin, respectively. The edematous lesions caused by edema toxin (7, 23, 25) have been attributed to the calmodulin-dependent, adenylate cyclase activity of EF (14, 15). Lethal toxin causes severe pulmonary edema and death in Fisher 344 rats (2, 7), is rapidly cytolytic for macrophages (8), and inhibits growth of certain cell lines (15). No enzymatic activity has been identified for LF. Both the lethal and edema toxins inhibit the release of superoxide anion (O_2^-) from polymorphonuclear leukocytes induced by bacterial products (32).

With our current knowledge of receptor mechanisms, early studies on anthrax toxin (14, 18) are now seen to be consistent with the hypothesis that PA must bind to receptors to permit subsequent binding of LF or EF. Protective antigen may also contain a common receptor site(s) for LF and EF, as demonstrated by competition between LF and EF in toxicity assays (6, 14, 24).

This report describes the preparation and characterization of monoclonal antibodies to PA. Two monoclonal antibodies were

obtained which neutralize lethal and edema toxin activity by inhibiting binding of PA to cell receptors.

MATERIALS AND METHODS

Bacillus anthracis antigens. Purified PA, LF, and EF antigens were prepared as previously described (14). Each protein was at least 95% pure as determined by gel electrophoresis, and contained < 0.1% contamination by other toxin components, as judged from toxicity assays.

Immunizations. Female BALB/c mice were immunized with purified PA by a different procedure for each fusion performed. Immunized mice used as spleen donors demonstrated high titers by ELISA for PA-reactive antibodies. See Table 1 for immunization schedules and procedures.

Hybridomas. Spleen cells from immunized mice were fused with either logarithmically growing SP2/0-Ag14 myeloma cells (5) for fusions PAI, PAII, PA2II, PA2III, and PA2IV or P3x-63-Ag8-653 myeloma cells for fusions PAIII and PAIV. The latter two fusions were performed by Hazelton Biotechnology Corporation, Vienna, Va. Hybridoma cultures were screened by the enzyme-linked immunosorbent assay (ELISA) method described below. Positive hybridomas were subcloned twice by limiting dilution and one to four clones for each hybridoma were expanded in vitro. Ascites from each hybridoma were produced by inoculating 1×10^6 hybridoma cells i.p. into BALB/c female mice 2-8 weeks after injection of 0.5 ml i.p. of pristane (2,6,10,14-tetramethyl pentadecane; Sigma Chemical Co., St. Louis, Mo). The ascites from each clone were pooled, clarified by centrifugation (2000 x g for 20 min), and stored at -70°C.

ELISA. The ELISA previously described (16) was used with a few changes: (i) dilutions were made in phosphate-buffered saline (PBS) with 0.5% gelatin and 0.05% Tween 20. (ii) Plates were incubated for 2 h at 37°C with rabbit antiserum to mouse immunoglobulins IgG, IgM, and IgA (Calbiochem-Behring, San Diego, Ca) diluted 1:400 prior to addition of horseradish peroxidase (HRP) conjugated to staphylococcal protein A (HRP-Protein A; Sigma). (iii) Incubation of HRP-Protein A was for 30 min at room temperature, and (iiii) wells with absorbance values > 0.200 nm were selected as positive wells.

Determination of antibody subclass. An ELISA-based, sub-type kit (Boehringer-Mannheim Biochemicals, Indianapolis, In) was used to determine immunoglobulin subtype and subclass specificity.

Affinities of Monoclonal Antibody. Apparent affinities of each purified monoclonal antibody (MAb) for PA were estimated from the slopes of ELISA binding curves (31) by using the ELISA described above instead of a radioimmunoassay (29). Goat antibody to mouse IgG and IgM conjugated to HRP (Kirkegaard and Perry, Gaithersburg, Md) at 1:1000 (0.5 µg/ml) was substituted for the rabbit antiserum to mouse IgG, IgM, and IgA and HRP-Protein A reagents in the ELISA assay above. Incubation was for 2 h at 37°C.

Purification of Monoclonal Antibody. Two to three ml of ascitic fluids from IgG monoclones were dialyzed against 20 mM Tris/HCl, pH 8.0; centrifuged (10,000 x g 30 min); and pumped onto a 14-ml column of DEAE Affi-Gel Blue (Bio-Rad, Rockville

Centre, NY). Elution was with a gradient of 60 ml each of 20 mM Tris/HCl, pH 8.0, and 20 mM Tris/HCl, pH 8.0, with 100 mM NaCl. Fractions (1.0 ml) were assayed by ELISA. The purified IgG was precipitated with 50% saturated $(NH_4)_2SO_4$, pH 7.5. The pellet was resuspended in water, extensively dialyzed against 10 mM Tris/HCl, pH 8.0, and frozen at -70°C.

Ascitic fluids containing IgM MAb were purified by the addition of $(NH_4)_2$ SO_4, pH 7.5, to 45% saturation. After 1 h, the material was centrifuged at 10,000 x g for 30 min, the pellet resuspended in 20 mM Tris/HCl (pH 8.0), and the precipitation repeated to 40% saturation. The precipitate was dissolved in and dialyzed extensively against PBS, pH 8.4 at 4°C and frozen at -70°C.

Determination of Ig concentration. Radial immunodiffusion plates were prepared with rabbit anti-mouse IgG (Miles Scientific, Naperville, Il) or rabbit anti-mouse IgM (Kirkegaard and Perry) in 0.5% agarose. Immunoglobulin concentrations were calculated from either IgG or IgM (Sigma) standard curves after incubation for 18-20 h (IgG) or 48 h (IgM) in a moist chamber at 23°C.

Isoelectric focusing. The monoclonal nature of each cell line was identified by isoelectric focusing (IEF). Purified immunoglobulins from IgG clones and marker proteins [IEF pI calibration kit (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ) or prestained IEF standards (Bio-Rad)] were analyzed on precast polyacrylamide gels, pH 3.5-9.5 (Pharamicia-LKB

Biotechnology, Inc.) according to the manufacturer's directions. Protein bands were visualized by Coomassie blue stain.

In vivo neutralization. Neutralization of lethal toxin was assayed in Fisher 344 male rats (225-250 g) by injection (i.v.) of mixtures of 40 μg of PA, 8 μg of LF, and 4 mg of protein of each monoclone after incubation at 37°C for 1 h. Protein concentrations were estimated by using the relationship of 1 A_{280} = 1 mg protein/ml. Only 1 rat per monoclone was tested initially. Ascites fluids that protected the rats or demonstrated a delayed time to death were retested at lower concentrations until no protection was observed. Four rats were tested for each ascitic fluid that neutralized the toxin.

In vitro neutralization assay. In the presence of edema toxin, Chinese hamster ovary (CHO) cells undergo an elongation in response to the adenylate cyclase activity of EF (14). Chinese hamster ovary cells, cultured in EMEM containing nonesential amino acids medium supplemented with 25 mM Hepes, 0.05 mg/ml gentamycin, and 10% fetal bovine serum, were plated thinly (3-6 x 10^4 cells per ml) in 96-well, flat-bottom, cell culture plates and incubated overnight before use. Monoclonal antibodies were serially diluted in separate 96-well plates containing 100 μl of toxin (100 ng of EF per ml, 50 ng of PA per ml) in cell culture medium. After 1 h at 37°C, 75 μl of the toxin-antibody mixture was transferred into the corresponding wells of a CHO cell plate. The final amounts per well were 8 ng EF and 4 ng PA. After incubation for 3 h at 37°C, the wells were inspected visually to

determine the smallest amount of MAb that inhibited CHO cell elongation.

Biotinylation of purified Monoclonal antibody. Purified MAb were biotinylated by a procedure modified from Stahli et al. (22). Briefly, MAb were adjusted to a concentration of 1 mg of IgG per ml after dialysis in 0.1 M $NaHCO_3$, 0.15 M NaCl, buffered to pH 8.5. N-Hydroxysuccinimidyl biotin (Sigma; 1 mg/ml in dimethylsulfoxide) was added (0.1 ml per ml of MAb) and incubated 3 h at room temperature. Biotinylated MAb were then dialyzed in 10 mM Tris/HCl, pH 8.0, and frozen at -70°C. For use, glycerol was added to 50% and vials held at -20°C.

Competitive binding assay. Competition between MAb for a single epitope was measured in an ELISA by using each purified MAb to compete for the binding of a limiting concentration of biotinylated MAb. Each blocking, non-biotinylated MAb (MAb_1) was used at a concentration which, in the regular ELISA (described above), gave an A_{405} value >2.0 while not giving significant, nonspecific A_{405} values on control plates lacking PA. After incubating 50 µl of MAb_1 for 1 h at 37°C, 50 µl of biotinylated purified MAb was added to each well (MAb_2), without removing unbound MAb_1, and the plate was incubated 2 h at 37°C. Biotin control wells contained of 50 µl of biotinylated, purified MAb and 50 µl PBS with 0.5% gelatin, 0.05% Tween 20. Wells were washed four times with PBS with 0.05% Tween 20. Avidin conjugated to HRP (Sigma) and diluted to 1:1000 (1 µg of avidin-HRP per ml) was added to each well (100 µl/well); plates were

incubated 1 h at 37°C. For color development, wells were washed six times with PBS with 0.05% Tween 20 and 100 µl of ABTS reagent [2,2'-Azino-bis(3-ethylbenzthiazolinesulfonic acid)] (Sigma), prepared as 1 mg reagent per ml in 0.1 M sodium citrate buffer (pH 4.0) and 0.003% hydrogen peroxide, was added to each well. The reaction was stopped after 15 min by adding 50 µl of 10% sodium dodecyl sulfate per well. Absorbance was read on a Dynatech Microelisa Auto Reader MR580 (Dynatech Instruments, Inc., Chantilly, Va) at a wavelength of 405 nm. Dilutions for MAb_2 were selected to give a minimum adsorption value of 0.300 for biotin control wells. The observed A_{405} values were used to calculate the percent that MAb_1 blocked the binding of the biotinylated MAb_2 in the following formula:

$$\% \text{ Blocking} = (1 - \text{test well } A_{405}/\text{control well } A_{405}) \times 100.$$

Selected MAb were also tested in a competitive binding assay similar to the procedure above except that MAb_1 was assayed on the ELISA microtiter plate prior to the addition of MAb_2.

Binding of ^{125}I-PA to FRL-103 cells. FRL-103 cells, plated in 24-well cell culture dishes in EMEM medium containing nonessential amino acids and supplemented with 10% fetal bovine serum, were grown to confluence. The monolayer was washed, and 0.40 ml of H199 medium, supplemented with 25 mM HEPES and 2% fetal bovine serum, and containing 0.2 µg of ^{125}I-PA per ml (2.4 x 10^7 cpm per µg) and individual ascities MAb (5 µg of Ig per ml)

was added to each well. Labeled PA, provided by A. M. Friedlander, was prepared by Bolton-Hunter reagent and retained biological activity. After incubation for 9 h at 0°C, the monolayers were washed four times with Hanks' balanced salt solution, dissolved in 1.0 ml of 0.1 M NaOH, and counted. Controls containing 30 μg nonradioactive PA per ml averaged 740 cpm, and wells with no competing MAb averaged 9000 cpm.

RESULTS AND DISCUSSION

This report describes the isolation and partial characterization of 37 MAb against the PA component of the toxin of B. anthracis. All PA MAb were specific for PA and did not bind to LF or EF by ELISA. Table 2 lists the 37 hybridoma clones, their apparent affinities, and Ig sub-types. Five separate fusions (PAI, PAII, PA2II, PA2III, and PA2IV) with the SP2/0 myeloma cell line resulted in 35 clones. Fusion experiments PAIII and PAIV, in which the P3x-63-ag8-653 myeloma cell line was used, resulted in only one clone each. Designations of clones in this report consist of the first three alphanumeric characters for each clone except for PA2II 2D3-1-1, designated as 2D3-1 to distinguish it from PAI 2D3-3-1 (2D3).

Thirty-one MAb were typed as IgG clones (24 IgG_1, 4 IgG_{2a}, and 3 IgG_{2b} subtype clones), and six typed as IgM clones (Table 2). All 37 clones contained the kappa light chain. We have no explanation why three of four clones in fusion PA2III were of the IgG_{2a} sub-type. The six IgM class hybridomas obtained from fusion PA2IV may have resulted from the i.p. and i.v. boosters administered on days -2 and -1. The MAb can be placed into one of three groups based upon their relative affinities (31): high affinity (affinity $\leq$ 0.2 μg), moderate affinity (affinity $\geq$ 0.2 μg to $\leq$ 1 μg), and low affinity (affinity $>$ 1 μg). The IgG_1 subtype MAb comprised 18 of 20 clones producing high affinity MAb. The remaining 17 clones produced moderate and low affinity MAb.

Isoelectric focusing of purified immunoglobulins from IgG hybridomas on polyacrylamide gels (fig. 1a and 1b) demonstrated banding pattern and pI value differences among the MAb. Only MAb 14B7 and 14C7 appeared to have identical pI's and IEF patterns (fig. 2). These two MAb were located in adjacent wells of the original 96-well fusion plate and contamination from one well into another probably occurred. MAb 3B6, which was functionally similar to 14B7 and 14C7 (discussed below), was different from them chemically, as shown by running these MAb individually and in mixtures on IEF gels (fig. 2). These results indicate that 36 individual PA hybridomas were obtained. We were unable to resolve adequately the isoelectric focusing patterns of the IgM clones by using agarose IEF.

In vivo neutralization studies showed a significant delay of time to death (2D3, 2D5) or survival (3B6, 14B7, 14C7) of Fisher 344 male rats after i.v. injection of mixtures of MAb ascites and PA+LF (fig. 3). Lethal toxin (40 μg PA, 8 μg LF) was neutralized by 4 mg, 0.4 mg, and 0.04 mg of 3B6, 14B7, and 14C7 ascites protein, respectively. Neutralization was confirmed with three more rats protected for each concentration of MAb ascites.

We observed neutralization of edema toxin (PA+EF) activity in CHO cells in vitro with 3B6, 14B7, and 14C7 (Table 3). A few other MAb (not listed) demonstrated an inhibition of edema toxin activity at undiluted or slightly diluted concentrations. Although this assay was not performed in a more quantitative

manner, such as measurement of cyclicAMP (14), we were able to observe by microscopy that these three MAb inhibited the cellular elongation response of CHO cells in the presence of edema toxin.

Analysis of epitope diversity among MAb has been described by others who used various competitive-binding assays (1, 3, 12, 13, 19, 20, 21, 26, 30, 31). A competitive-binding ELISA assay, with biotinylated MAb and fixed concentrations of unlabeled competing MAb, allowed us to designate at least 23 different antigenic regions on the PA molecule (Table 4). The distinctive binding features of 3B6 and 14B7 were further demonstrated in a competitive-binding ELISA with biotinylated 3B6 or 14B7 and varying concentrations of several representative, unlabeled, competing MAb (fig. 4 and 5). The data from the competitive-binding experiments also indicated that 3B6, 14B7, and 14C7 recognized the same antigenic region on the PA molecule. These preliminary results will aid us in the study of the various binding regions of the PA molecule.

Monoclonal antibodies 3B6, 14B7, and 14C7 inhibited binding of ^{125}I-PA to the receptor site on FRL-103 cells by 97.5%, 95.1%, and 96.5%, respectively; whereas the controls and the other MAb ranged from 0-49.2%. The antigenic region of PA recognized by 3B6, 14B7, and 14C7 appears to be the cell receptor-binding site(s), thus explaining the neutralizing ability of these three clones against the activity of either lethal or edema toxin. These results support previous proposals that binding of PA to

its cell receptor is required prior to expression of lethal or edema toxin activity. It is probable then that PA acts as active B-fragment containing receptor-binding activity and LF and EF act as active A-fragments containing biological activity in the usual A-B enzymatic-binding structure of intracellular bacterial protein toxins described by Gill (9).

In summary, we have developed and characterized 36 different MAb against the PA component of anthrax toxin. Two of these MAb were able to neutralize the lethal and edema toxin acitvities by inhibiting the binding of PA to cell receptors, thus providing supporting evidence that initial binding of PA to cell receptors is required for toxic activity.

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LITERATURE CITED

1. Adam, E., I. Nasz, A. Lengyel, J. Erdei, and J. Fachet. 1987. Determination of different antigenic sites on the adenovirus hexon using monoclonal antibodies. Arch. Virol. 93:261-271.

2. Beall, F. A., M. J. Taylor, and C. B. Thorne. 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of Bacillus anthracis. J. Bacteriol. 83:1274-1280.

3. Carbone, F. R. and Y. Paterson. 1985. Monoclonal antibodies to horse cytochrome C expressing four distinct idiotypes distribute among two sites on the native protein. J. Immunol. 135:2609-2616.

4. Cianfriglia, M., D. Armellini, A. Massone, and M. Mariani. 1983. Simple immunization protocol for high frequency production of soluble antigen-specific hybridomas. Hybridoma 2:451-457.

5. Earley, E. M., and M. C. Osterling. 1985. Fusion of mouse-mouse cells to produce hybridomas secreting monoclonal antibody. J. Tissue Culture Meth. 9:141-146.

6. Ezzell, J. W., B. E. Ivins, and S. H. Leppla. 1984. Immunoelectrophoretic analysis, toxicity, and kinetics of in

vitro production of the protective antigen and lethal factor components of Bacillus anthracis toxin. Infect. Immun. 45:761-767.

7. Fish, D. C., B. G. Mahlandt, J. P. Dobbs, and R. E. Lincoln. 1968. Purification and properties of in vitro-produced anthrax toxin components. J. Bacteriol. 95:907-918.

8. Friedlander, A. M. 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. 261:7123-7126.

9. Gill, D. M. 1978. Seven toxic peptides that cross cell membranes, p. 291-332. In J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, N.Y.

10. Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins. 1985. Demonstration of a capsule plasmid in Bacillus anthracis. Infect. Immun. 49:291-297.

11. Hambleton, P., J. A. Carman, and J. Melling. 1984. Anthrax: the disease in relation to vaccines. Vaccine 2:125-132.

12. Huber-Lukac, M., F. Jaquet, P. Luethy, R. Huetter, and D. G. Braun. 1986. Characterization of monoclonal antibodies to a crystal protein of Bacillus thuringiensis subsp. kurstaki.

Infect. Immun. 54:228-232.

13. Huber-Lukac, M., P. Lüthy, and D. G. Braun. 1983. Specificities of monoclonal antibodies against the activated delta-endotoxin of Bacillus thuringiensis var. thuringiensis. Infect. Immun. 40:608-612.

14. Leppla, S. H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. Proc. Natl. Acad. Sci. U.S.A. 79:3162-3166.

15. Leppla, S. H., B. E. Ivins, and J. W. Ezzell, Jr. 1985. Anthrax toxin, p.63-66. In L. Leive (ed.), Microbiology-85. American Society for Microbiology, Washington, D.C.

16. Little, S. F., and G. B. Knudson. 1986. Comparative efficacy of Bacillus anthracis live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. Infect. Immun. 52:509-512.

17. Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Dreier. 1983. Evidence for plasmid-mediated toxin production in Bacillus anthracis. Infect. Immun. 39:371-376.

18. Molnar, D. M., and R. A. Altenbern. 1963. Alterations in the biological activity of protective antigen of Bacillus

anthracis toxin. Proc. Soc. Exp. Bio. Med. 114:294-297.

19. Ray, K., J. Vockley, and H. Harris. 1984. Epitopes of human intestinal alkaline phosphatases, defined by monoclonal antibodies. FEBS Lett. 174:294-299.

20. Sheppard, A. J., D. Cussell, and M. Hughes. 1984. Production and characterization of monoclonal antibodies to tetanus toxin. Infect. Immun. 43:710-714.

21. Smith-Gill, S. J., T. B. Lavoie, and C. R. Mainhart. 1984. Antigenic regions defined by monoclonal antibodies correspond to structural domains of avian lysozyme. J. Immunol. 133:384-393.

22. Stahli, C., V. Miggiano, J. Stocker, Th. Staehelin, P. Haring, and B. Takacs. 1983. Distinction of epitopes by monoclonal antibodies. Meth. Enzymol. 92:242-253.

23. Stanley, J. L., K. Sargeant, and H. Smith. 1960. Purification of Factors I and II of the anthrax toxin produced in vivo. J. Gen. Microbiol. 22:206-218.

24. Stanley, J. L., and H. Smith. 1961. Purification of Factor I and recognition of a third factor of the anthrax toxin. J. Gen. Microbiol. 26:49-66.

25. Thorne, C. B., D. M. Molnar, and R. E. Strange. 1960. Production of toxin in vitro by Bacillus anthracis and its separation into two components. J. Bacteriol. 79:450-455.

26. Tzartos, S. J., D. E. Rand, B. L. Einarson, and J. M. Lindstrom. 1981. Mapping of surface structures of electrophorus acetylcholine receptor using monoclonal antibodies. J. Biol. Chem. 256:8635-8645.

27. Turnbull, P. C. B., M. B. Broster, J. A. Carman, R. J. Manchee, and J. Melling. 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect. Immun. 52:356-363.

28. Uchida, I., R. Sekizaki, K. Hashimoto, and N. Terakado. 1985. Association of the encapsulation of Bacillus anthracis with a 60 megadalton plasmid. J. Gen. Microbiol. 131:363-367.

29. Van Heyningen, V., D. J. H. Brock, and S. Van Heyningen. 1983. A simple method for ranking the affinities of monoclonal antibodies. J. Immunol. Meth. 62:147-153.

30. Volk, W. A., B. Bizzini, R. M. Snyder, E. Bernhard, and R. R. Wagner. 1984. Neutralization of tetanus toxin by distinct monoclonal antibodies binding to multiple epitopes on the toxin

molocule. Infect. Immun. **45**:604-609.

31. **Wnek, A. P., R. J. Strouse, and B. A. McClane.** 1985. Production and characterization of monoclonal antibodies against Clostridium perfringens type A enterotoxin. Infect. Immun. **50**:442-448.

32. **Wright, G. G., and G. L. Mandell.** 1986. Anthrax toxin blocks priming of neutrophils by lipopolysaccharide and by muramyl dipeptide. J. Exp. Med. **164**:1700-1709.

TABLE 1. Immunization of female BALB/c mice with purified protective antigen

	Immunization Route	PA (μg)	Diluent[a] (0.5 ml)	Injection Schedule
Fusion I	i.m.	20	FCA	Day 0
	i.m.	20	FIA	17 and 29 weeks
	i.v.	50	Saline	40 weeks (3 days PF[b])
Fusion II	i.m.	20	FCA	Day 0
	i.m.	20	FIA	29 weeks
	i.v.	50	Saline	61 weeks (3 days PF)
Fusion III	i.m.	1	FCA	Day 0
	i.m.	10	FIA	3 and 10 weeks
	i.m.	10	IFA	35 weeks (3 days PF)
Fusion IV	i.p.	50	FCA	15 and 8 days PF
	i.p.	5	Saline	3 days PF
	i.p. and i.v.	2.5	Saline	2 and 1 days PF
Fusion 2II	i.p.	50	FCA	Day 0 and 7
	i.p.	5	Saline	Day 12
	i.p. and i.v.	2.5	Saline	Day 13 and 14
	i.v.	20	Saline	8 weeks
	i.v.	100	Saline	25 weeks (day 3 PF)
Fusion 2III	i.m.	10	FCA	Day 0
	i.m.	10	FIA	4 weeks
	i.v.	100	Saline	16 weeks (day 3 PF)
Fusion 2IV	i.m.	10	FCA	Day 0
	i.m.	10	FIA	4 weeks
	i.p.	200	Saline	16 weeks (day 3 PF)
	i.p.	100	Saline	Day 2 PF
	i.p.	100	Saline	Day 1 PF

[a] FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant.

[b] PF = pre-fusion

TABLE 2. Affinities and sub-types of 37 PA monoclonal antibody-producing hybridoma clones raised in seven separate fusions.

Fusion	Hybridoma Clone	Affinity (μg)	Antibody Sub-type
PAI	1G7-2-1	0.064	G_1
	2D3-3-1	0.033	G_1
	2D5-1-1	0.048	G_1
	3B6-1-1	0.521	G_1
	3D2-1-1	0.079	G_1
	3F3-2-2	0.070	G_1
	3F10-1-1	0.044	G_{2b}
	6C3-1-1	0.054	G_1
PAII	1C5-1-1	0.115	G_1
	2D3-1-1	0.054	G_1
	2G4-1-1	0.144	G_1
	3C5-1-1	0.210	G_1
	7C3-1-1	0.098	G_1
PAIII	6B7-1-1	275	G_1
PAIV	1F2-1-1	0.047	G_1
PA2II	2F7-1-1	20	G_{2b}
	2F9-1-1	0.070	G_1
	3D11-1-1	0.103	G_{2a}
	10D2-1-1	0.036	G_1
	10G4-1-1	0.047	G_1
	14B7-1-1	0.069	G_1
	14C7-1-1	0.107	G_1
	16E11-2-1	0.051	G_1
	18C2-1-1	0.399	G_1
	18G8-2-1	0.421	G_1
	20C5-1-1	0.092	G_1
PA2III	2B8-1-1	0.377	G_{2a}
	8C8-1-1	0.389	G_{2a}
	9B4-2-1	0.228	G_{2a}
	9E11-1-1	20	G_1
PA2IV	3E2-1-1	2.9	M
	4F3-2-1	4.7	M
	5B3-2-1	4.7	M
	5G3-1-1	37	G_{2b}
	6F3-1-1	8.9	M
	16E5-1-1	6.3	M
	16E9-1-1	0.945	M

TABLE 3. Neutralization of edema toxin activity in CHO cells by PA monoclonal antibody[a].

Monoclone	Ascitic fluid Ig (μg)	Purified Ig (μg)
3B6	6 ± 3.7	8
14B7	23 ± 35	1
14C7	9 ± 13.8	5

[a] Edema toxin contained 4 ng of PA and 8 ng of EF. Calculated average values and standard deviations of known IgG concentrations and effective dilution of either ascitic fluid Ig (four experiments for each monoclone) or purified MAb Ig (two experiments for each monoclone).

TABLE 4. Determinants on the PA molecule identified by EISA blocking assays.

Determinant	Monoclone	Determinant	Monoclone
1	1G7,2D3,2D5,3D2,	12	18C2
	3C5,2F9,10D2,10G4	13	18G8
2	3B6,14B7,14C7	14	2B8
3	3F3,3F10,6C3	15	8C8
4	1C5,2D3-1	16	9E11
5	2G4,20C5	17	3E2
6	7C3	18	4F3
7	6B7	29	5B3
8	1F2	20	5G3
9	2F7	21	6F3
10	3D11,9B4	22	16E5
11	16E11	23	16E9

FIGURE LEGENDS

FIG. 1a & 1b. Isoelectric focusing patterns of purified IgG from PA MAb separated on polyacrylamide isoelectric focusing gels.

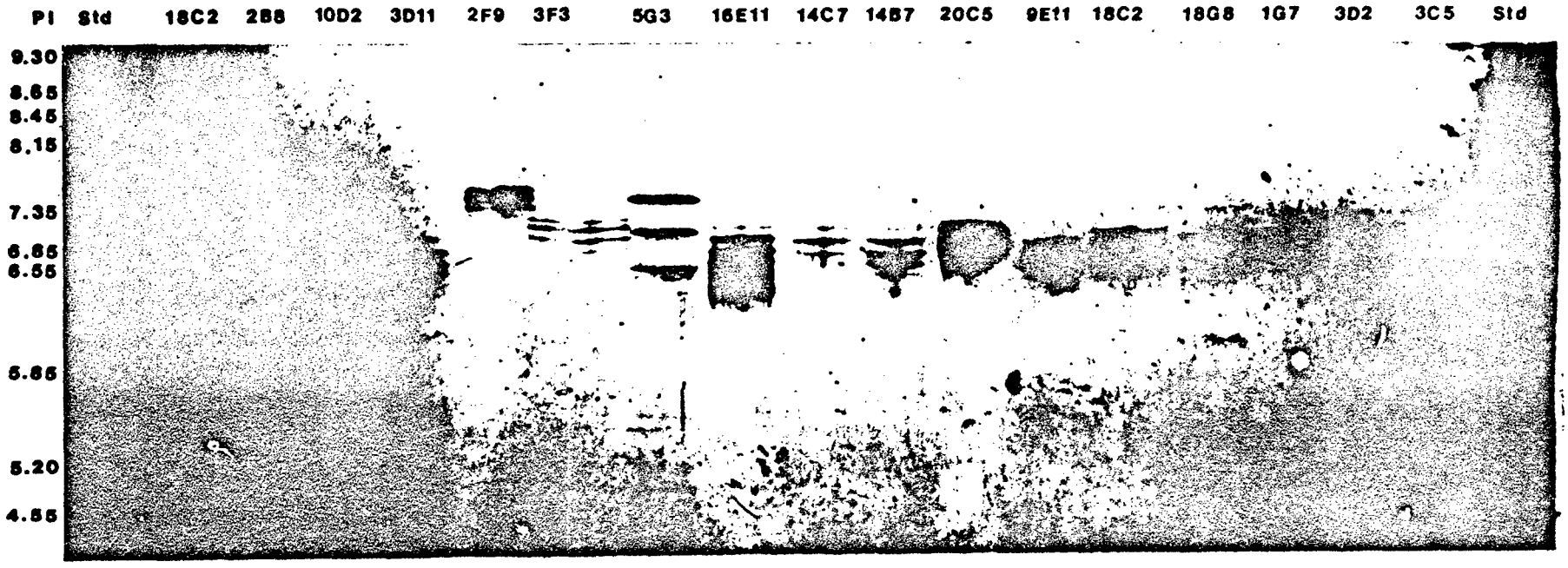
FIG. 2. Isoelectric focusing patterns of purified IgG from MAb 3B6, 14B7, and 14C7 individually and in mixtures separated on polyacrylamide isoelectric focusing gels.

FIG. 3. Survival of Fisher 344 rats injected i.v. with 40 μg PA + 8 μg LF. Survival groups consist of four guinea pigs each. All other groups consist of one to two guinea pigs.

FIG. 4. Competitive antibody-binding assay with biotinylated and nonlabeled MAb. The binding of biotinylated 3B6 was measured in the presence of different concentrations of competing, unlabeled MAb; (■) 14B7, (▼) 3B6, (✕) 3F3, (▲) 9E11, (◆) 1C5, and (+) 2D3.

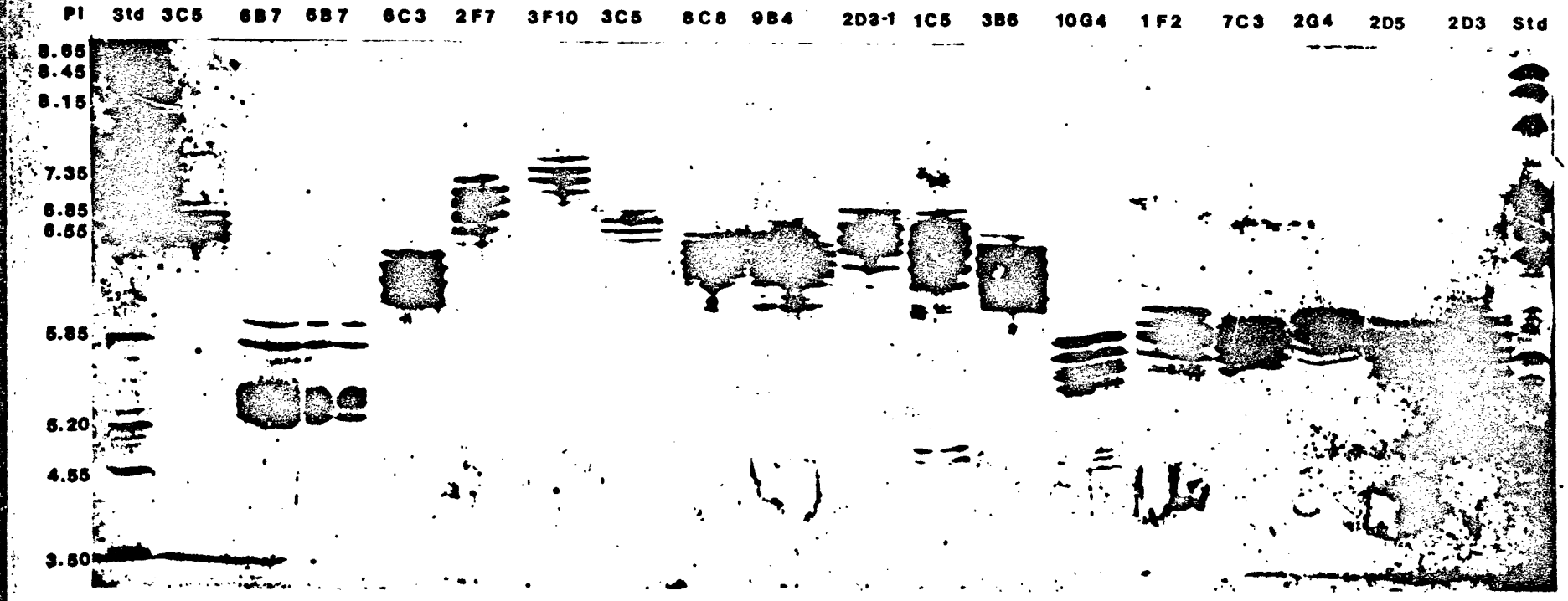
FIG. 5. Competitive antibody-binding assay with biotinylated and nonlabeled MAb. The binding of biotinylated 14B7 was measured in the presence of different concentrations of competing, unlabeled MAb; (■) 14B7, (▼) 3B6, (✕) 2B8, (▲) 6B7, (◆) 2F9, and (+) 16E9.

CATHODE (-)



ANODE (+)

CATHODE (-)



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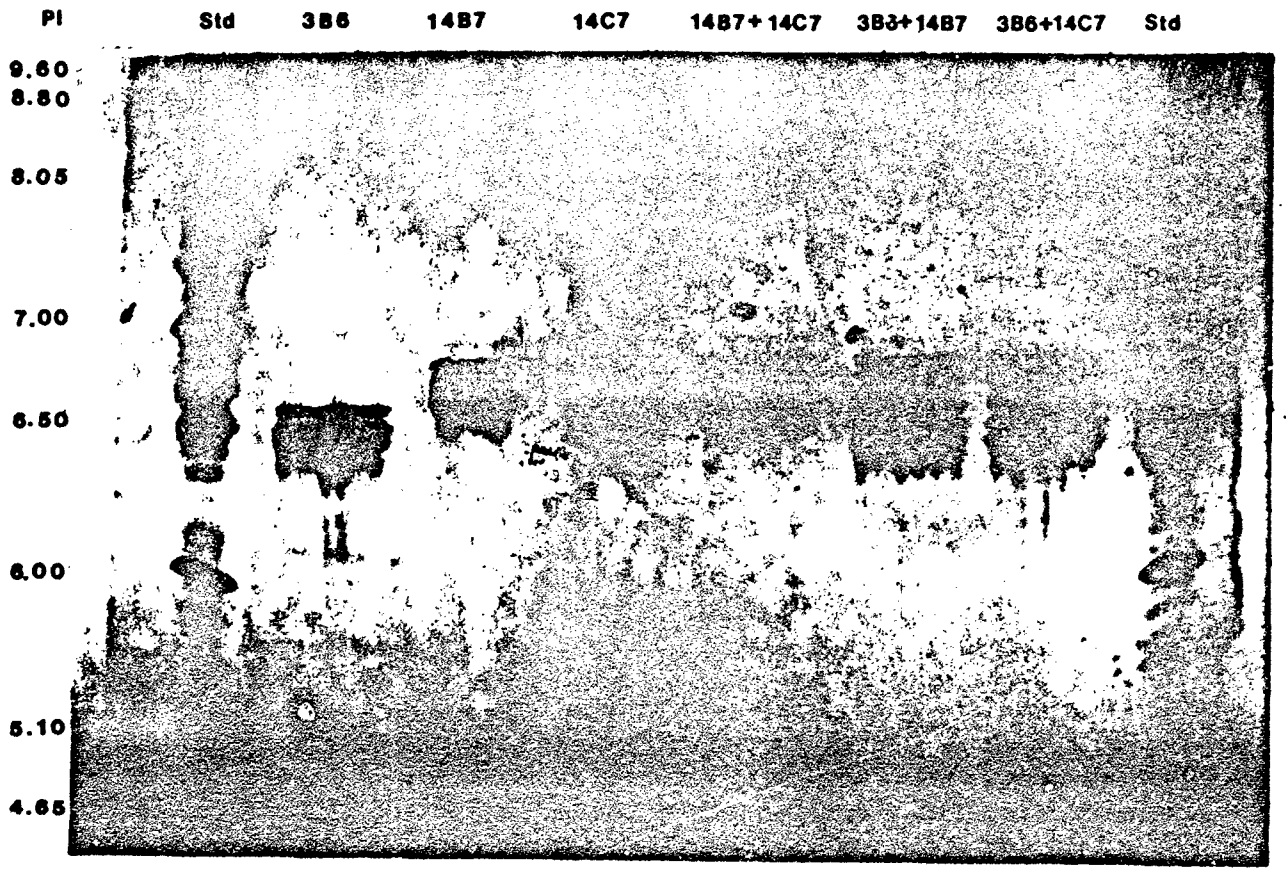

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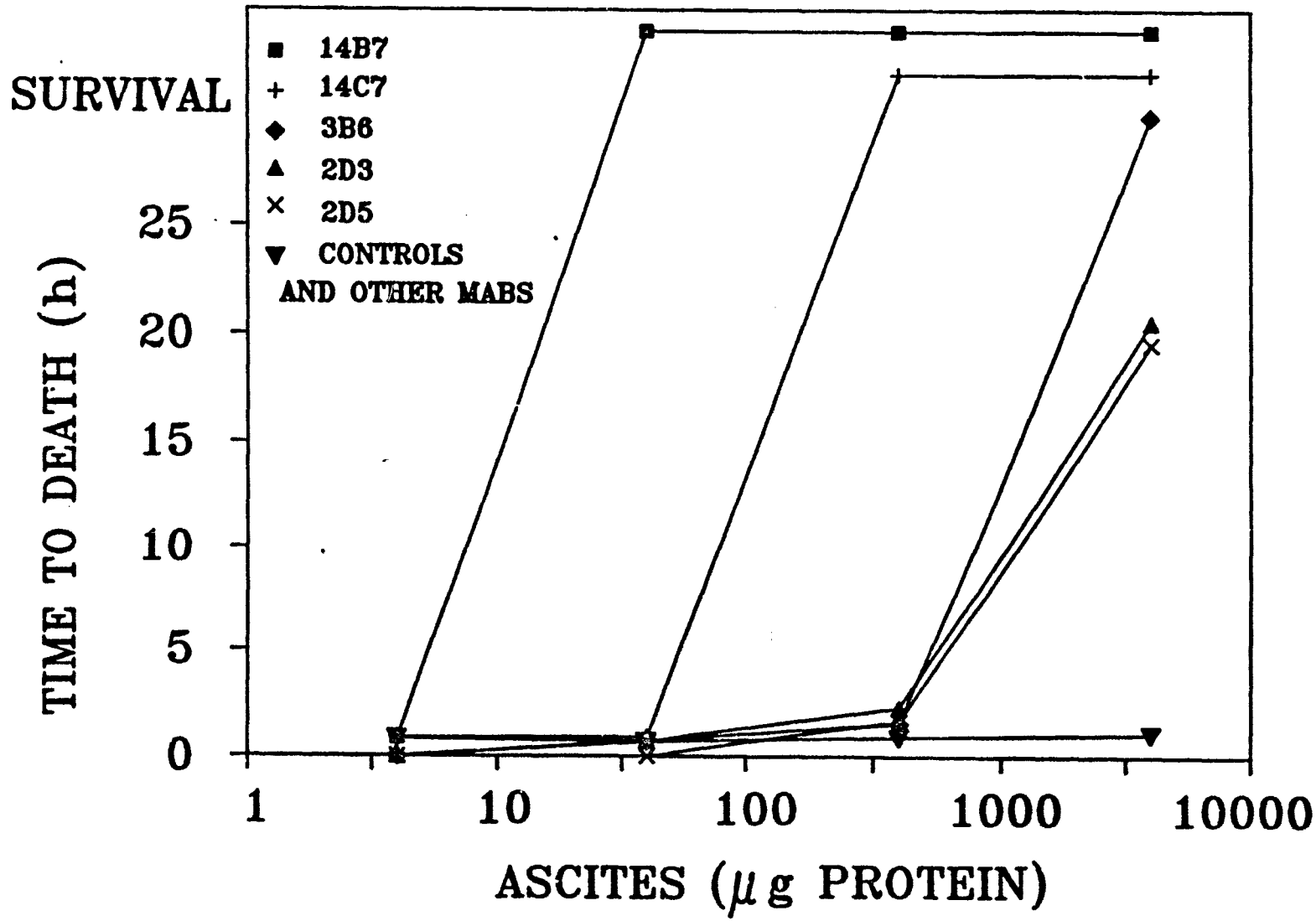
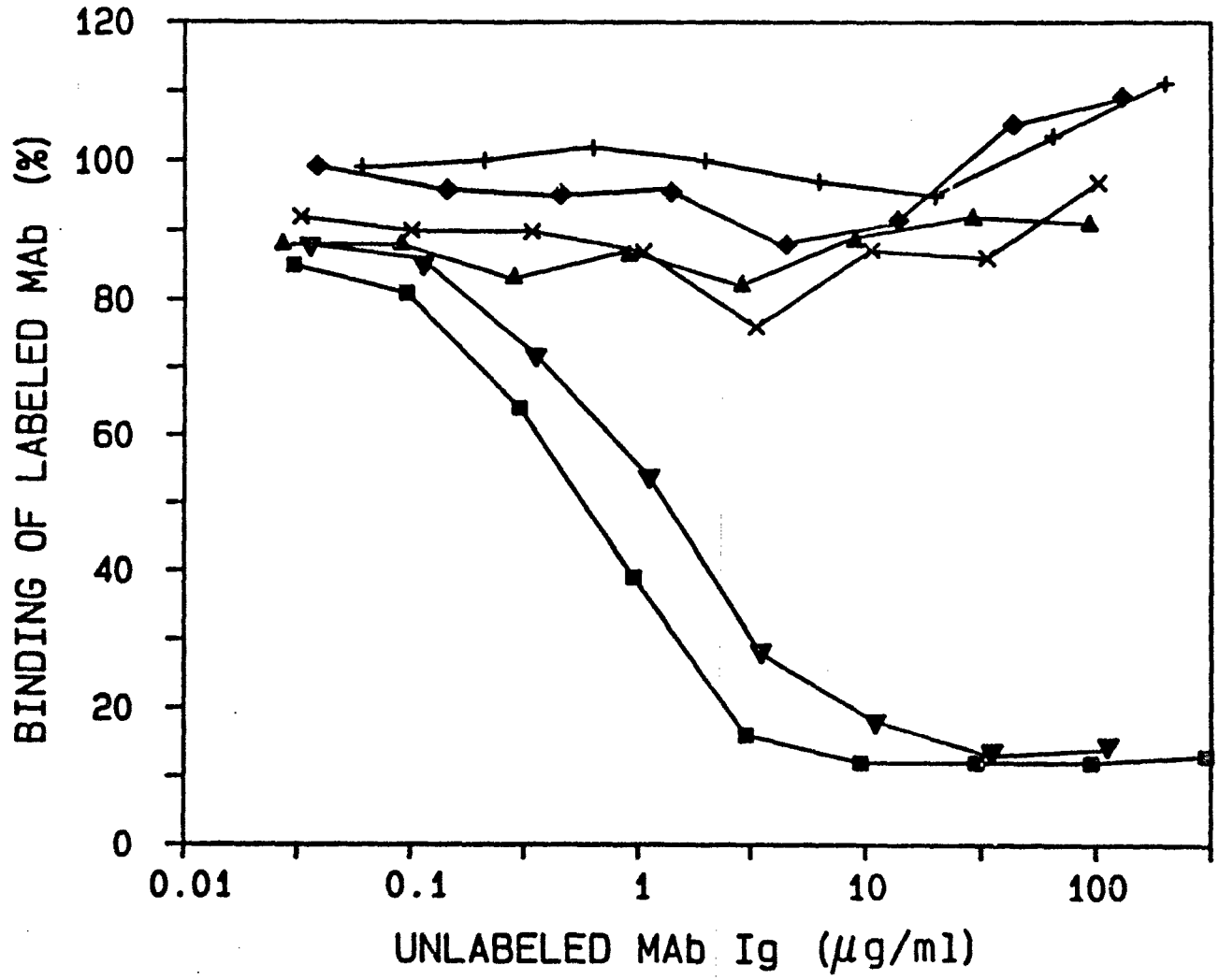
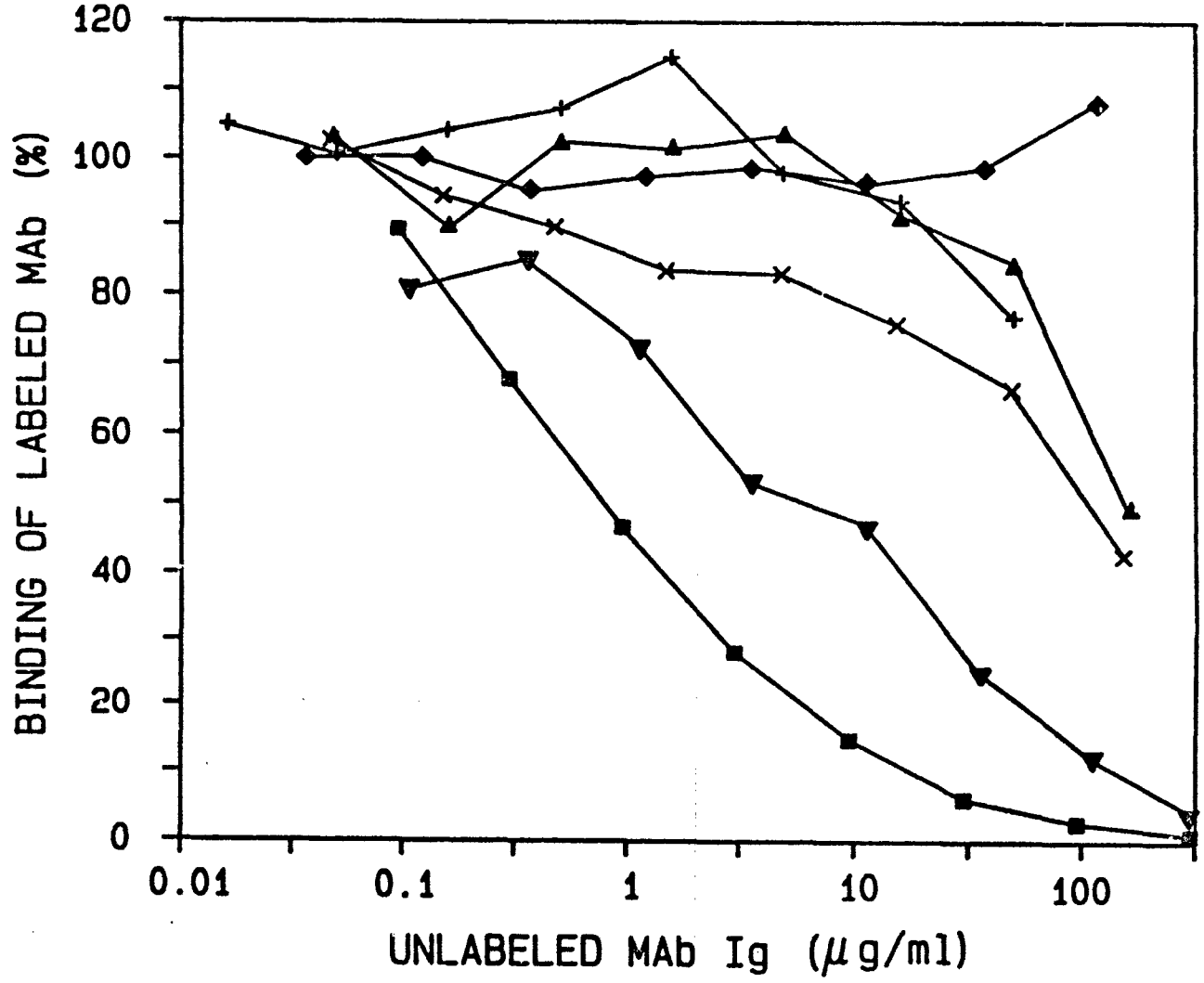

SURVIVAL
TIME TO DEATH (h)
25
20
15
10
5
0
1
10
100
1000
10000
ASCITES (μg PROTEIN)
14B7
14C7
3B6
2D3
2D5
CONTROLS
AND OTHER MABS


120
100
80
60
40
20
0
BINDING OF LABELED MAb (%)
0.01
0.1
1
10
100
UNLABELED MAb Ig (μg/ml)


120
100
80
60
40
20
0
BINDING OF LABELED MAb (%)
0.01
0.1
1
10
100
UNLABELED MAb Ig (μg/ml)

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Thirty-six monoclonal antibodies to the protective antigen protein of Bacillus anthracis exotoxin have been characterized for affinity, antibody sub-type, competitive binding to antigenic regions, and ability to neutralize the lethal and edema toxin activities. At least 23 antigenic regions were detected on protective antigen by a blocking, enzyme-linked immunosorbent assay. Two clones, 3B6 and 14B7, competed for a single antigenic region and neutralized the activity of both the lethal toxin in vivo (Fisher 344 rat) and the edema toxin in vitro (CHO cells). (cont)

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20. ABSTRACT (cont)

These two antibodies blocked the binding of ^{125}I-PA to FRL-103 cells. Our results support the proposal that binding of protective antigen to cell receptors is required for expression of toxicity.